Reports of Biochemistry & Molecular Biology
Vol.8, No.4, Jan 2020

Original article

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Molecular Detection of *Adefg* Efflux Pump Genes and their Contribution to Antibiotic Resistance in *Acinetobacter baumannii* Clinical Isolates

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Abstract

Background: *Acinetobacter baumannii (A. baumannii)* is one of the most important bacteria causing nosocomial infections worldwide. Over the past few years, several strains of *A. baumannii* have shown antibiotic resistance, which may be due to the activity of efflux pumps. This study was aimed to detect *AdeFG* efflux pump genes and their contribution to antibiotic resistance in *A. baumannii* clinical isolates.

Methods: A total of 200 *A. baumannii* clinical isolates were collected from clinical specimens of ulcers, pus, sputum, and blood. All isolates were identified using standard biochemical tests. After identifying and cleaving the genome by boiling, PCR was performed on samples using specific primers. The antimicrobial susceptibility patterns were determined by disk diffusion, with and without CCCP efflux pump inhibitor were determined according to CLSI guidelines.

Results: We identified 60 clinical isolates of *A. baumannii* using biochemical differential tests. Identification of all *A. baumannii* isolates was confirmed by blaOXA-51-like PCR. According to the results of our study, 98.37% of *A. baumannii* isolates were resistant to ciprofloxacin, norfloxacin, and levofloxacin. PCR results indicated that all 60 *A. baumannii* isolates contained the *AdeF* and 76.66% contained *AdeG*.

Conclusions: the results of this study demonstrated that most of the *A. baumannii* isolates contained *AdeF* and *AdeG efflux pump genes*, and more than 98% of the isolates were resistant to ciprofloxacin, norfloxacin, and levofloxacin. This reflected the significant contribution of efflux pumps to the development of resistance to these antibiotics.

Keywords: *Acinetobacter baumannii, AdeFG,* Antibiotic Resistance, Efflux pump, Molecular detection.

Introduction

Acinetobacter baumannii (*A. baumannii*), a gram-negative bacterium in cocci or coccobacillus shapes without fermentation potential (1-3), can be isolated from patients and many environmental sources (4). These bacteria have minimal nutritional requirements for growth and can survive in adverse conditions including both dry and aquatic environments. *A. baumannii* is rarely the cause of severe infections in individuals with normal immunity and is rarely found in in healthy individuals (5). *A. baumannii* is the leading cause of nosocomial infections, including as pneumonia, meningitis, and urinary and respiratory tract infections. This pathogen also appears in burn wound infections and in patients admitted to intensive care units (ICUs) (6-8). *A. baumannii* has intrinsic resistance to a variety of antibiotics and is also highly prone to acquiring antimicrobial resistance (9, 10). The antibiotic-resistance property of this pathogen causes many problems for the treatment of *A. baumannii* infections (11). Acinetobacter strains may also reveal multidrug resistance (MDR) properties (12, 13). Resistance to antibiotics in this bacterium is caused by various

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Received: 24 Dec, 2019; Accepted: 19 Mar, 2020

mechanisms, including antibiotic inactivation, target modification, and changes in membrane permeability, as well as several other factors including outer membrane proteins, production of diverse groups of β-lactamases, production of altering enzymes for aminoglycosides, and production of a multidrug efflux pump (14-16). Efflux pumps contribute to intrinsic and acquired resistance in *A. baumannii* to a wide range of antibiotics and disinfectants (17). This is due to the increased expression of efflux pump genes through mutation of this antimicrobial resistance (18). Resistance-nodulation-cell division (RND) efflux pumps are an important efflux pump family that contributes to drug resistance in *A. baumannii* strains. In the RND pump genes, three efflux pumps, *AdeABC*, *AdeIJK*, and *AdeFGH* have been identified in Acinetobacter species (4, 10, 19). *AdeFGH* expression is regulated by the LysR-type transcriptional regulator (LTTR) and confers MDR when highly expressed (20). *AdeFGH* has been found in about 90% of clinical isolates and its high expression is responsible for high levels of resistance to chloramphenicol, clindamycin, fluoroquinolones, and trimethoprim (19). The present study was aimed to investigate the expression of *AdeFG* efflux pump genes in clinical isolates of *A. baumannii*, and their role in the development of antibiotic resistance in Tehran province.

Materials and methods

We collected 200 clinical isolates of *A. baumannii* from clinical specimens of ulcers, pus, sputum, and blood in Shahid Mostafa Khomeini, Tohid, and Shahid Motahari hospitals of Tehran province in 2017. All samples were transferred to the laboratory in brain heart infusion (BHI) medium. Acinetobacter isolates were identified using standard biochemical methods, including the oxidase test, MacConkey agar medium, incubation at 37 and 42 °C, triple sugar iron (TSI) agar, citrate utilization, motility and urea tests, gelatin and arginine hydrolysis, oxidase, Dnase, and glucose-containing OF media (21, 22). The blaOXA-51-like gene was used to confirm *A. baumannii*. The following two specific primers with PCR product size of 342 bp were used to identify the blaOXA-51-like gene: Oxa-51-like-F 5-TAATGCTTTGATCGGCCTTG-3 and Oxa-51-like-R 5-TGGATTGCACTTCATCTTGG-3. After primer-BLAST on designated primers, their sensitivity and specificity were determined by NCBI site, and PCR was performed for *A. baumannii*. The PCR program included a denaturation step at 95 °C for 5 min, with 30 cycles of denaturation at 95 °C for 45 seconds, annealing at 58 °C for 60 seconds, and extension at 72 °C for 60 seconds, with a final extension at 72 °C for 5 minutes. The PCR products were electrophoresed. The antimicrobial susceptibility patterns were determined according to the CLSI guidelines by Kirby-Bauer disk diffusion for the antibiotics norfloxacin, ciprofloxacin, and levofloxacin (MAST, UK), for which efflux pumps produce resistance in *A. baumannii*. DNA was extracted from the clinical samples through boiling and PCR was performed with specific primers for *AdeF* and *AdeG*. Characteristics of the selected primers are presented in Table 1. After primer-BLAST on the primers, their sensitivity and specificity were determined via the NCBI site, and the PCR was performed for *A. baumannii*. The volumes were 25 µl per reaction. The PCR program included a denaturation step at 94 °C for 5 min, with 30 cycles of denaturation at 94 °C for 60 seconds, annealing at 56 °C for 60 seconds, and extension at 72 °C for 60 seconds, with a final extension at 72 °C for 7 min. Finally, the PCR products were electrophoresed.

Table 1. Sequence of the study primers.

Gene	Primers sequence	Product size	Reference
AdeF	F: GGTGTCGACCAAGATAAACG R: GTGAATTTGGCATAGGGACG	207	23
AdeG	F: TTCATCTAGCCAAGCAGAAG R: GTGTAGTGCCACTGGTTACT	468	23

Results

Specimen collection and culture

Of 200 collected *A. baumannii* clinical isolates, 60 were identified using biochemical differential tests. Identification of all the *A. baumannii* isolates was confirmed by blaOXA-51-like PCR. Of these, 25 samples were isolated from the ICU, 17 from the infectious diseases ward, 13 from the emergency department, and 5 from other areas of the hospital. Of these, 25 were from patient blood samples (41.7%), 15 from sputum (25%), 12 from ulcers (20%), and 8 from pus (13.3%).

Determination of antimicrobial susceptibility patterns using disk diffusion

Disk diffusion showed that 59 of the 60 (98.34%) *A. baumannii* samples were resistant to all three antibiotics (Table 2). This reflected the significant contribution of efflux pumps to the development of resistance to these antibiotics.

Table 2. Resistance of *A. baumannii* isolates to different antibiotics.

Number and percentage of resistant isolates	Antibiotics
59 (98.34)	**Ciprofloxacin**
59 (98.34)	**Norfloxacin**
59 (98.34)	**Levofloxacin**

PCR results for AdeF and AdeG genes

After the antibiotic-resistance assessment of the isolates, the AdeF and AdeG frequencies were determined by PCR. The AdeF and AdeG PCR products were then electrophoresed electrophoresed (Figs. 1 and 2). Of the 60 *A. baumannii* isolates, all 60 contained AdeF and 46 contained AdeG (Table 3).

Table 3. The frequency of *AdeFG* efflux pump genes in *A. baumannii* strains.

Number of isolates with efflux pump genes	Percentage of isolates with efflux pump genes	Genes
60	100	***AdeF***
46	76.66	***AdeG***
46	76.66	***AdeFG***

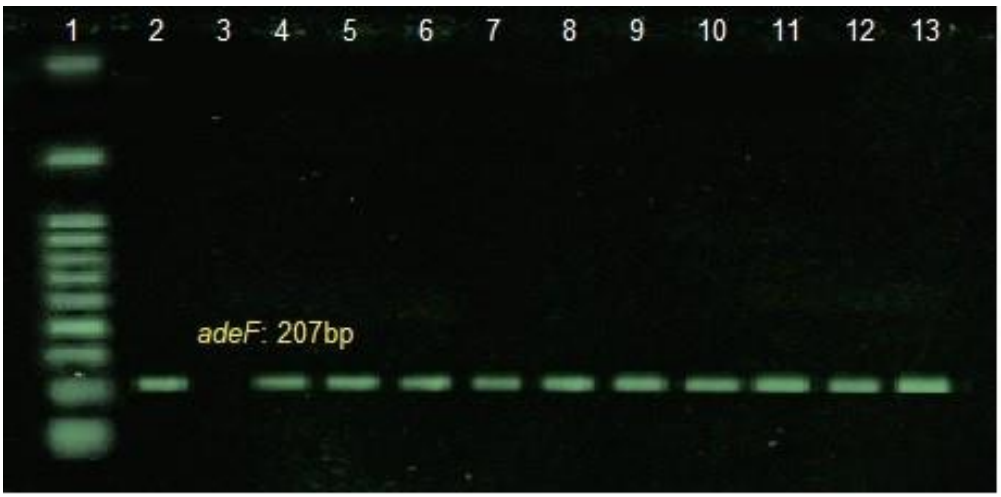

Fig. 1. PCR amplification of the *AdeF* gene. Lane 1: Ladder (100 bp), lane 2: positive control (207bp); lane 3: negative control, lane 4- 12, and 13: positive results.

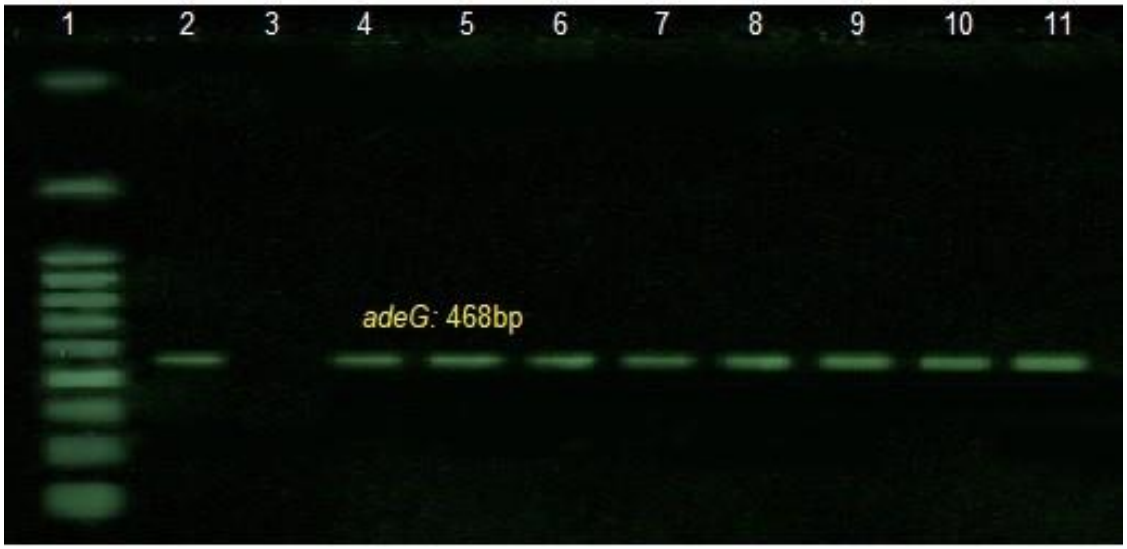

Fig. 2. PCR amplification of the *AdeG* gene. Lane 1: Ladder (100 bp), lane 2: positive control (468bp); lane 3: negative control, lane 4- 10, and 11: positive results.

Discussion

Acinetobacter species, especially *A. baumannii*, which have both intrinsic and acquired antibacterial resistance and high tendency to possess antibiotic-resistance genes are common causes of nosocomial infections, especially in ICUs (24). The selected drugs for treatment of infections caused by these bacteria are generally broad-spectrum penicillin and carbapenems, to which this species has acquired increased resistance (25). Carbapenem-resistant *A. baumannii* strains have become a major global concern because these resistant strains are very difficult to treat (26). Antibiotic efflux pumps are one of the most widespread mechanisms for developing resistance among microorganisms. Efflux pumps are present in both pathogenic and opportunistic Acinetobacter species. Thus, identification and prevalence assessment of these pumps are of great importance in the treatment of bacterial infections (10, 27). Antibiotic efflux pumps remove antibiotics from bacteria, thereby reducing their therapeutic effect (28). This study found that a high percentage of the *A. baumannii* isolates were resistant to ciprofloxacin, norfloxacin, and levofloxacin. Consistent with our results, Goodarzi and colleagues examined the expression and function of efflux pump genes in MDR *A. baumannii* isolates, and reported the antibiotic resistance varying from 45-98.3%. Of their isolates, 90% contained AdeI and AdeJ, indicating the association between these genes and antibiotic resistance (29).

Daimier-Piolle and colleagues found that 100% of their MDR *A. baumannii* isolates contained the AdeIJK efflux pump gene. All the isolates were resistant to at least one antibiotic with the following breakdown: gentamicin (45%), amikacin (96.7%), imipenem (70%), meropenem (66.7%), ceftazidime (96.7%), ciprofloxacin (56.7%), trimethoprim-sulfamethoxazole combination (55%), tetracycline (98.3%), and ceftriaxone (83.3%). In this study, AdeI and AdeJ of the *A. baumannii* RND system were investigated as target genes by PCR, and the frequency of these two genes in the examined isolates was found to be 90%. The expression of AdeIJ was consistent with its resistance to tetracycline, ceftriaxone, trimethoprim sulfamethoxazole, ciprofloxacin, and ceftazidime (30), which is consistent with the results of our study. Maliki et al. examined *A. baumannii* strains isolated from burn wound infections in Tehran province, and found that 100% of the isolates were ciprofloxacin resistant (31), which was also consistent with our study. Ardebili and colleagues also investigated the relationship between ciprofloxacin resistance due to the AdeABC efflux system in *A. baumannii* clinical isolates and concluded that 95.6% of their isolates were ciprofloxacin resistant (32), which also agreed

with our study. Our results indicate that resistance to ciprofloxacin, norfloxacin, and levofloxacin exists in AdeFG gene-carrying strains due to the efflux pump, and they have a significant relationship.

Acknowledgment

This manuscript is part of a MSc thesis. The authors express their gratitude to all microbiology laboratory staff of Shahed University of Medical Sciences.

References

1. Khayat H, Sadeghifard N, Pakzad I, Azimi L, Somayeh Delfani S, Sayehmiri K, et al. Determination of Different Fluoroquinolone Mechanisms Among Clinical Isolates of *Acinetobacter baumanniiin* in Tehran, Iran. Iran Red Crescent Med J. 2017; 19(9): 58798.
2. Esmaeilzadeh Ashini E, Amini k. Isolation of tetD, tetC, tetB, tetA genes from *Acinetobacter bummani* samples isolated from hospital samples by multiplex PCR method. Medical Journal of Mashhad University of Medical Sciences. 2018; 60(6): 734-742.
3. Jasim HN, Hafidh RR, Abdulamir AS. Formation of therapeutic phage cocktail and endolysin to highly multi-drug resistant *Acinetobacter baumannii*: *in vitro* and *in vivo* study. Iran J Basic Med Sci. 2018; 21(11):1100-1108.
4. JaponiNejad AR, Sofian M, Ghaznavi-Rad E. Molecular detection of *adeABC* efflux pump genes in clinical isolates of *Acinetobacter baumannii* and their contribution in imipenem resistance. Iran South Med J 2014: 17(5): 815-823.
5. Ghalebi M, Eslami G, Zandi H, Farhang A, Vakili M, Mohammadi N, Dehghan Banadkouki A. Survey of Antibiotic Resistance and Frequency of *blaOXA-23* and *blaOXA-24* Oxacillinase in *Acinetobacter baumannii* Isolated from Tracheal Tube Specimens of Patients Hospitalized in Intensive Care Units in Isfahan city. J Shahid Sadoughi Univ Med Sci. 2017; 25(1): 1-10.
6. Karbasizade V, Yousefian R, Moghim Sh. Identification and Frequency of Colistin Resistant *Acintobacter baumanii* from Clinical Isolates by PCR. J Isfahan Med Sch. 2014; 32(301):1-9.
7. Ansari H, Tahmasebi-Birgani M, Bijanzadeh M, Doosti A, Kargar M. Study of the immunogenicity of outer membrane protein A (ompA) gene from *Acinetobacter baumannii* as DNA vaccine candidate in vivo. Iran J Basic Med Sci. 2019; 22(6):669-675.
8. Niakan M, Kaviani R, Heidari Motlagh R, Pouladi I. Investigation of the resistance of *Acinetobacter baumanii* strains isolated from Tehran's hospital inpatients in terms of selected antibiotics. Journal of Basic and Clinical Pathophysiology. 2018; 6(2):13-16.
9. Rahimzadeh A, Farajnia S, Pourbabaee MA, Ansarin Kh, Zolfaghari MR, Masoudi N. Detection of Prevalence of *OXA-2* and *OXA-10* Type ESBL and Class I Integron among *Acintobacter baumannii* Strains Isolated from Patients of Tabriz City (Iran) by PCR Technique. J Babol Univ Med Sci. 2012; 14(5): 56-63.
10. Dostvandi S, Abiri R, Mohajeri P, Alvandi A. Genotypic and Phenotypic Characteristics of Efflux Pumps in *Acinetobacter spp*. Isolated from Inpatients. Journal of Mazandaran University of Medical Sciences. 2015; 25(126): 1-10.
11. Wybo I, Blommaert L, De Beer T, Soetens O, De Regt J, Lacor P, et al. Outbreak of multidrug-resistant *Acinetobacter baumannii* in a Belgian university hospital after transfer of patients from Greece. J Hosp Infect. 2007; 67(4): 374-380.
12. Choi JY, Park YS, Kim CO, Park YS, Yoon HJ, Shin SY, et al. Mortality risk factors of *Acinetobacter baumannii* bacteremia. Inter Med J. 2005; 35(10): 599-603.
13. Madadi-Goli N, Moniri R, Bagheri-Josheghani S, Dasteh-Goli N. Sensitivity of levofloxacin in combination with ampicillin-sulbactam and tigecycline against multidrug-resistant *Acinetobacter baumannii*. Iran J Microbiol. 2017; 9(1):19-25.
14. El Kettani A, Maaloum F, Diawara I, Katfy K, Harrar N, Zerouali K, Belabbes H, Elmdaghri N. Prevalence of *Acinetobacter baumannii* bacteremia in intensive care units of Ibn Rochd University Hospital, Casablanca. Iran J Microbiol. 2017; 9(6):318-323.
15. Van Bambeke F, Balzi E, Tulkens PM. Antibiotic efflux pump. Biochem Pharmacol. 2000; 60(4): 457-470.
16. Huang L, Sun L, Xu G, Xia T. Differential susceptibility to carbapenems due to the *adeABC* efflux pump among nosocomial outbreak isolates of

Acinetobacter baumannii in a Chinese hospital. Diagnostic microbiology and infectious disease. 2008; 62(3):326-32.
17. Schneiders T, Findlay J, Amyes SG. Efflux Pumps in *Acinetobacter baumannii*. Acinetobacter Biology and Pathogenesis. 2008:105-127.
18. Coyne S, Courvalin P, Périchon B. Efflux mediated antibiotic resistance in *Acinetobacter spp*. Antimicrobial agents and chemotherapy. 2011; 55(3):947-953.
19. Rosenfeld N, Bouchier C, Courvalin P, Périchon B. Expression of the resistance-nodulation-cell division pump *adeIJK* in *Acinetobacter baumannii* is regulated by *adeN*, a TetR-type regulator. Antimicrobial agents and chemotherapy. 2012; 56(5):2504-2510.
20. Nitzan Y, Pechatnikov I, Bar-El D, Wexler H. Isolation and characterization of heat-modifiable proteins from the outer membrane of Porphyromonas asaccharolytica and *Acinetobacter baumannii*. Anaerobe. 1999; 5(1):43-50.
21. Wieczorek P, Sacha P, Hauschild T, Zórawski M, Krawczyk M, Tryniszewska E. Multidrug resistant *Acinetobacter baumannii*–the role of *adeABC* (RND family) efflux pump in resistance to antibiotics. Folia Histochem Cytobiol. 2008; 46(3):257-267.
22. Constantiniu S, Romaniuc A, Iancu LS, Filimon R, Taraşi I. Cultural and biochemical characteristics of *Acinetobacter spp*. Strains isolated from hospital units. J Prevent Med. 2004; 12(3-4): 35- 42.
23. Coyne S, Rosenfeld N, Lambert T, Courvalin P, Perichon B. Overexpression of Resistance-Nodulation-Cell Division Pump *adeFGH* Confers Multidrug Resistance in *Acinetobacter baumannii*. Antimicrobial agents and chemotherapy. 2010; 54(10): 4389- 93.
24. Szabo D, Szentandrassy J, Juhasz Z, Katona K, Nagy K, Rokusz L. Importand PER-1 producing Psedomonas aeruginosa, PER-1 producing *Acinetobacte baumannii* and *VIM2*- producing Pseudomonas aeruginosa strains in Hungary. Ann Clin Microbiol Antimicrob. 2008; 7: 12.
25. Katragkou A, Roilides E. Successful treatment of multidrug-resistant *Acinetobacter baumannii* central nervous system infections with colistin. J Clin Microbiol. 2005; 43(9): 4916-4917.
26. Ruiz M, Marti S, Fernandez Cuenca F, Pascual A, Vila J. Prevalence of IS(Aba1) in epidemiologically unrelated *Acinetobacter baumannii* clinical isolates. FEMS microbiology letters. 2007; 274(1):63-6.
27. Najar Peerayeh SH, Esmaeili D. Antibiotic Efflux Pumps. Ann Mil Health Sci Res. 2004; 2(1): 301-306.
28. Putman M, van Veen HW, Konings WN. Molecular properties of bacterial multidrug transporters. Microbiol Mol Biol Rev. 2000; 64(4): 672-693.
29. Goudarzi H, Doraghi M, Dabiri H, Ghalavand Z. Functional analysis of multidrug efflux pumps genes of *Acinetobacter baumannii* strains**.** Research in Medicine. 2013; 37(2): 107-112.
30. Damier-Piolle L, Magnet S, Brémont S, Lambert T, Courvalin P. AdeIJK, A resistance-nodulation-cell division pump effluxing multiple antibiotics in *Acinetobacter baumannii*. Antimicrobial agents and chemotherapy. 2008; 52(2):557-562.
31. Maleki MH, Jalilian FA, Khayat H, Mohammadi M, Pourahmad F, Asadollahi Kh, et al. Detection of highly ciprofloxacin resistance *Acinetobacter baumannii* isolated from patients with burn wound infections in presence and absence of efflux pump inhibitor. Maedica-a Journal of Clinical Medicine. 2014; 9(2):162-167
32. Ardebili A ,Lari AR, Talebi M. Correlation of ciprofloxacin resistance with the *adeABC* efflux system in *Acinetobacter baumannii* clinical isolates. Annals of laboratory medicine. 2014; 34(6):433-438.